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Ubiquitination of α5β1 Integrin Controls Fibroblast Migration through Lysosomal Degradation of Fibronectin-Integrin Complexes

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SUMMARY

Cell migration requires endocytosis and recycling of integrins, but it is not known whether degradation of these membrane proteins is involved. Here we demonstrate that in migrating cells, a fraction of the endocytosed fibronectin receptor, α5β1 integrin, is sorted into multivesicular endosomes together with fibronectin and degraded in lysosomes. This sorting requires fibronectin-induced ubiquitination of the α5 subunit, and the activity of the endosomal sorting complex required for transport (ESCRT) machinery, which interacts with α5β1 integrin. Importantly, we demonstrate that both α5 ubiquitination and ESCRT functions are required for proper migration of fibroblasts. We propose that ligand-mediated degradation of α5β1 integrin via the ESCRT pathway is required in order to prevent endosomal accumulation of ligand-bound integrins that might otherwise form nonproductive adhesion sites. Fibronectin and α5β1 integrin therefore are trafficked to lysosomes in a similar way to growth factors and their receptors.

INTRODUCTION

Cell migration is important for embryonic development, wound healing, and immune responses and also plays a role in pathological processes including cancer, vascular disease, osteoporosis, chronic inflammatory diseases, and mental retardation (Ridley et al., 2003). It is therefore crucial to understand the mechanisms that regulate the cell's ability to move.

Cell migration is characterized by a series of events that initiate by a front-to-back polarization in response to extracellular signals (Ridley et al., 2003). Next, an actin-rich lamellipodium extends from the cell body onto a cell surface (Nobes and Hall, 1999), while the tail of the cell is contracted. At the leading edge, new adhesion points to the substrate form, termed adhesion sites, which are endocytosed at the trailing edge, allowing the cell to detach. While this is the classic view of integrin recycling, it has been established that recycling loops in fact occur much more locally, controlled by the small GTPases Rab4, Rab11, or Rab25 (Caswell et al., 2007). This constitutes cycles of adhesion and detachment, and the turnover of adhesion sites determines the ability of a cell to migrate. The main component of adhesion sites is integrins, which are type I transmembrane glycoproteins consisting of α and β chains (Hynes, 2002). These heterodimers are noncovalently associated cell-surface receptors and form the mechanical link between the extracellular matrix (ECM) and the cytoskeleton.

By binding to ECM ligands (e.g., fibronectin, vitronectin, or collagen), integrins are activated and able to regulate many aspects of cell behavior by initiating intracellular cascades of signaling. ECM turnover is thought to occur via extracellular degradation of ECM proteins by matrix metalloproteinases (MMPs), plasmin, and other proteases (Marchina and Barlati, 1996; Shapiro, 1998), but also via internalization of ECM proteins and degradation in lysosomes (Godyna et al., 1995; Memmo and McKeown-Longo, 1998; Murphy-Ullrich and Mosher, 1987; Wienke et al., 2003).

It has been shown that β1 integrin endocytosis plays an important role in turnover of matrix fibronectin (Shi and Sottile, 2008), suggesting that β1 integrin and fibronectin are trafficked together, at least during endocytosis. While it is widely accepted that fibronectin is degraded via lysosomes (Sottile and Chandler, 2005), it has not been investigated whether integrins and fibronectin are trafficked together to this organelle.

Past views of integrin trafficking in cell migration proposed a back-to-front recycling of endocytosed molecules (Bretscher, 1989; Caswell and Norman, 2006; Pellinen and Ivaska, 2006; Ridley et al., 2003). However, there is in fact little evidence for this type of long-range transport during cell migration, but rather during cytokinesis, where integrins are redistributed en masse to the cleavage furrow (Pellinen et al., 2008). Integrins are internalized at the plasma membrane via several types of endocytosis and are recycled either via the long-recycling loop dependent on Rab11 or via the short recycling loop in a Rab4-dependent manner (Caswell et al., 2009). Whether the integrins that recycle are bound to ECM ligands and therefore are activated remains to be determined. So far, integrin trafficking has been studied

independently of ECM turnover, and it therefore remains unclear which pools of integrins are trafficked via which routes.

The fibronectin receptor, α5β1 integrin, is highly expressed in human fibroblasts and promotes their motility and survival (Lee and Juliano, 2000; Wary et al., 1996; Zhang et al., 1995). It has been shown to localize to early endosomes (Bretscher, 1989) and Rab4 and Rab11 positive recycling endosomes (Roberts et al., 2001), as well as to multivesicular endosomes (MVEs) (Ng et al., 1999). The localization of β1 integrin to the lumen of enlarged endosomes induced by the overexpression of Rab21 has been observed (Pellinen et al., 2006), but the functional significance of this localization has not been investigated. Even though integrins might be recycled back from MVEs to the plasma membrane, the localization of proteins within the lumen of MVEs is usually associated with the trafficking of proteins on their way to lysosomal degradation, a process that is well characterized for growth factor receptors (Gruenberg and Stenmark, 2004). This raises the question whether integrins are degraded via the lysosomal pathway, and whether their degradation has any consequence for cell migration.

In this article, we focus on the intracellular trafficking of α5β1 integrin. We demonstrate that this integrin dimer is ubiquitinated in migrating cells in response to fibronectin binding and degraded in lysosomes by a mechanism involving the endosomal sorting complex required for transport (ESCRT) machinery (Hurley and Emr, 2006; Raiborg and Stenmark, 2009). Fibronectin, which has been described to be also degraded via lysosomal degradation, is trafficked together with α5β1 integrin toward lysosomes. This orchestrated degradation is required for proper fibroblast migration, revealing an unexpected role for degradative integrin trafficking in cell motility.

RESULTS

Fibronectin, α5, and β1 Integrin Localize to Multivesicular Endosomes in Fibroblasts

In order to investigate the intracellular localization of fibronectin and α5β1 integrin in migrating cells, we fixed and prepared BJ normal human fibroblasts for cryo-electron microscopy (EM), and sections were labeled with antibodies against fibronectin, α5 integrin, and β1 integrin. Integrin labeling was observed on the plasma membrane (Figure 1A) and typical early endosomes containing few internal vesicles (Figure 1B for α5 integrin, Figure 1C for β1 integrin). Labeling was also observed on MVE-like structures of varying sizes, with small MVEs showing the densest labeling (Figure 1D). Fibronectin labeling was most prominent on extracellular deposits along the plasma membrane (Figure 1E) and in the endoplasmic reticulum (Figure 1F), consistent with the fact that fibroblasts produce fibronectin themselves. MVEs were also positive for fibronectin (Figure 1F). The fact that we observed fibronectin together with α5 integrin in MVEs (Figure 1G) suggests that fibronectin and a portion of α5β1 integrin are trafficked together from endosomes to lysosomes, possibly on their way to degradation. Interestingly, colocalization between fibronectin and α5 integrin was mostly observed in MVEs, while some early endosomes labeled exclusively for integrin. We hypothesize that integrin present without fibronectin in these early endosomes corresponds to recycling integrins on their way back to the plasma membrane, while integrin that is together with fibronectin corresponds to active ligand-bound integrin.

Interestingly, labeling by immunofluorescence of integrin at the plasma membrane in BJ cells was relatively weak compared with other cell types such as HeLa (see Figure S1 available online). This could be explained by the fact that fibroblast cells are more motile than HeLa cells (data not shown), and that highly motile cells might require more trafficking of integrins and therefore contain a higher intracellular proportion.

α5 Integrin Is Ubiquitinated in Response to Fibronectin Binding and This Promotes Its Degradation

As a prerequisite for their lysosomal degradation, cell-surface receptors are sorted within the lumen of MVEs into intraluminal vesicles (ILVs), usually in a ligand-dependent fashion. This allows the segregation of the cytoplasmic domain of the receptor, which is involved in signaling, thereby interfering with its ability to signal (Gruenberg and Stenmark, 2004; Katzmann et al., 2002). Ubiquitin acts as signal for the sorting of endocytosed membrane proteins into the lumen of MVEs, a process that requires the ubiquitin-binding ESCRT machinery (Hurley and Emr, 2006; Raiborg and Stenmark, 2009). Ubiquitination predominantly occurs at lysine residues in the cytoplasmic domain of membrane proteins, and is catalyzed by E3 ubiquitin ligases (Haglund and Dikic, 2005). Interestingly, the short cytoplasmic tails of α5 and β1 integrin contain several lysine residues. To investigate whether α5 and β1 integrin are ubiquitinated, we performed immunoprecipitation of endogenous α5 or β1 in HEp2 cells transfected with HA-tagged ubiquitin (Figure 2A; data not shown). HEp2 cells were used for biochemical studies requiring overexpression since BJ fibroblasts show low transfection efficiency only sufficient for microscopy studies. We observed a HA-ubiquitin-containing smear in lanes where α5 integrins was immunoprecipitated, suggesting that α5 integrin is poly- or multiubiquitinated, presumably at its cytoplasmic lysine residues.

In order to confirm that α5 integrin is ubiquitinated at cytoplasmic lysines, a mutant in which all four cytoplasmic lysines (labeled red in Figure S2A) were replaced by arginines was constructed and transfected into HEp2 cells, in addition to HA-tagged ubiquitin. We observed much less ubiquitination in the mutant-transfected cells (α5-4XR-GFP) compared with the wild-type GFP fusion protein of α5 integrin (α5-WT-GFP), confirming that ubiquitination of α5 integrin occurs at one or more of its conserved lysine residues (Figure 2B). The residual smear in α5-4XR-GFP-expressing cells is probably due to interaction of α5-4XR-GFP with β1 integrin, which is itself ubiquitinated (data not shown).

The detected ubiquitination of α5 integrin raised the question whether this posttranslational modification occurs constitutively or is induced. Knowing that fibronectin is a ligand for α5β1 integrin, we incubated HEp2 cells, which do not produce fibronectin themselves (Figure S2B), with or without fibronectin in serum-free medium, allowing a robust fibronectin matrix to be established. We observed an ubiquitination smear of α5 integrin in cells that were incubated with fibronectin, but not in cells treated with medium alone (Figure 2C). This indicates that α5β1 integrin is ubiquitinated in response to binding to its ligand fibronectin, which is reminiscent of the ligand-induced ubiquitination of growth factor receptors (Levkowitz et al., 1998, 1999).

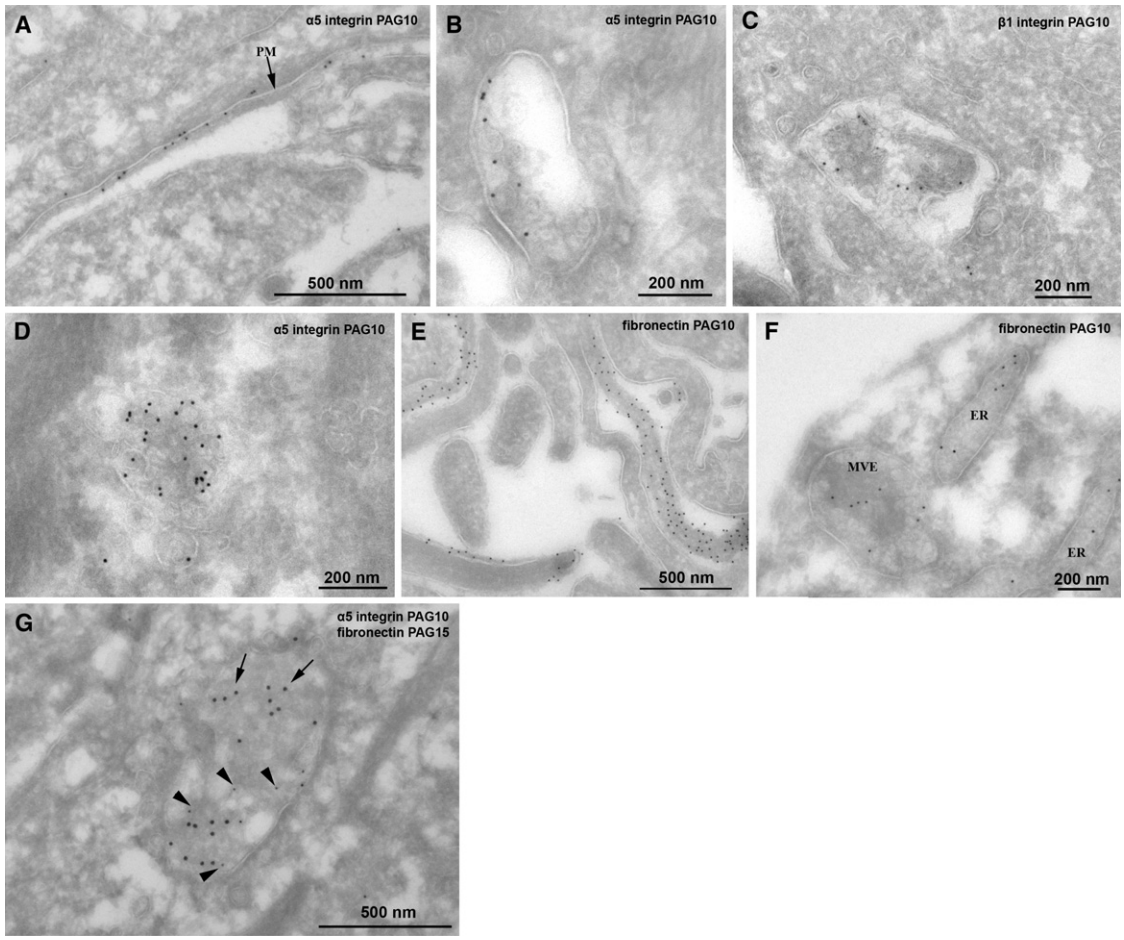

Figure 1. Fibronectin, α5, and β1 Integrin Localize to MVEs in Fibroblasts

BJ fibroblasts were prepared for cryo-electron microscopy and stained with antibodies against fibronectin, α5, and β1 integrin. Immunolabeling was performed as described in Experimental Procedures. Integrin labeling was observed on the plasma membrane (PM) (A) and in typical early endosomes containing few internal vesicles (B and C). (B) and (C) depict α5 and β1 integrin labeling, respectively. Importantly, we also found labeling on MVE-like structures of varying sizes (D). Fibronectin labeling was most prominent on extracellular deposits along the PM (E) and in the endoplasmic reticulum (ER) (F). As with α5 and β1 integrin, MVEs were also positive for fibronectin (F). (G) represents colocalization between α5 integrin (arrowheads) and fibronectin (arrows). Scale bars as indicated. See also Figure S1.

Since it is established that ubiquitinated cargo interacts directly with some of the ESCRT components that have ubiquitin-binding domains (Hurley and Emr, 2006; Raiborg and Stenmark, 2009), we next investigated whether α5β1 integrin binds to the ESCRT machinery. These experiments were conducted in BJ cells since we focused on interactions between endogenous proteins. When integrin was immunoprecipitated using an antibody against β1 integrin and the western blot probed with antibodies against Tsg101 or integrins, we observed that Tsg101 coimmunoprecipitated with endogenous α5 and β1 integrin (Figure 2D). This indicates that a fraction of α5β1 is in complex with ESCRT-I.

We also determined recycling rates (via the perinuclear recycling compartment, PNRC) and degradation rates of α5β1 integrin, and observed that α5β1 integrin recycling occurs over the course of minutes (Figure 2E), whereas degradation occurs over the course of several hours (Figure 2F). We observed that α5-WT-GFP has a half-life of approximately 24 hr, while α5-4XR-GFP was much less degraded over this time (Figure 2F). This prolonged half-life of the mutant protein is consistent with the finding that α5-WT-GFP is ubiquitinated and therefore degraded faster than α5-4XR-GFP, which is not ubiquitinated (Figure 2B). Endogenous α5 integrin had a half-life of 18 hr (Figure S2C), which is shorter than that of α5-WT-GFP presumably due to the fact that this experiment was performed in BJ cells, which produce fibronectin themselves, and therefore integrin may be ubiquitinated at a higher extent, leading subsequently to quicker degradation. Due to their different dynamics, integrin recycling and degradation are seemingly two processes that occur separately, suggesting that it is a subset of integrins that is trafficked via the lysosomal degradation route, presumably corresponding to the molecules that have been bound to fibronectin.

Even though lysine to arginine substitution is a highly conservative amino acid substitution not expected to interfere with most protein functions, we confirmed that the substitutions introduced in the cytoplasmic tail of α5 integrin do not interfere with

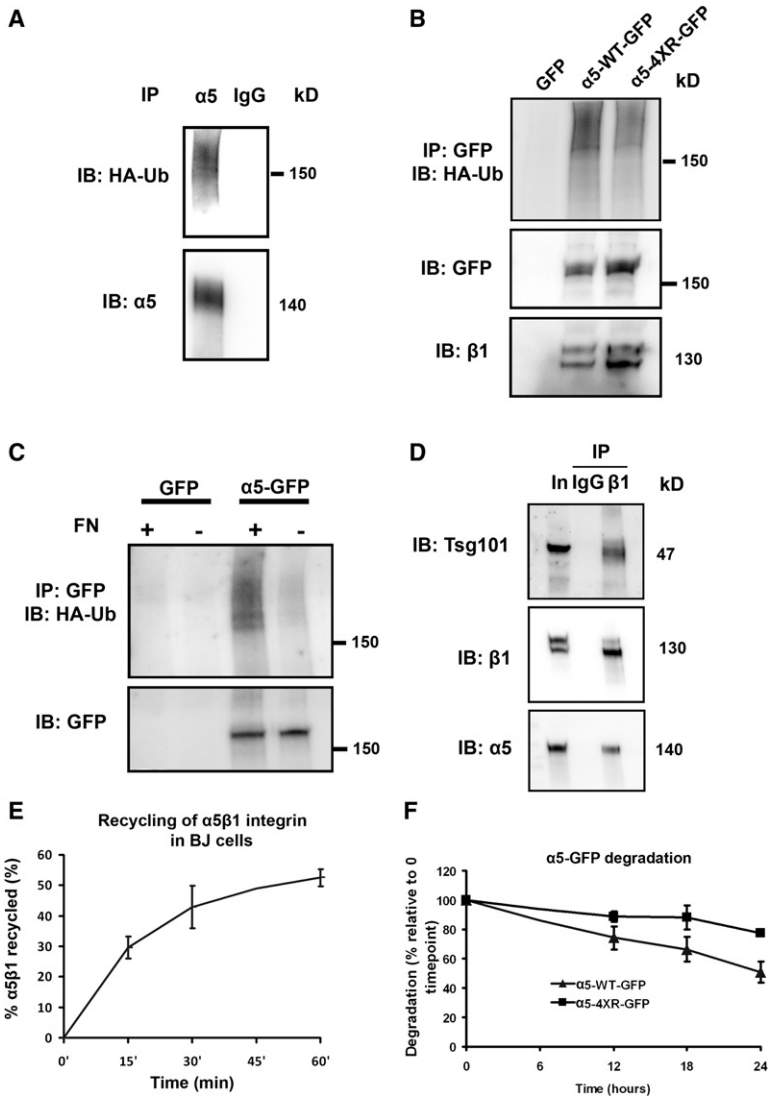

Figure 2. α5β1 Integrin Is Ubiquitinated upon Binding to Fibronectin, which Promotes Its Degradation

(A) HEp2 cells were transfected with HA-Ubiquitin (HA-Ub) for 24 hr and immunoprecipitation was performed using anti-α5 antibody. Ubiquitination was detected by immunoblotting with anti-HA antibody.

(B) HEp2 cells were transfected with HA-Ubiquitin and GFP, α5-WT-GFP, or α5-4XR-GFP for 24 hr. Immunoprecipitation was performed using GFP trap beads, membranes were immunoblotted with anti-HA to detect ubiquitination, and loading was checked using anti-GFP antibody and β1 integrin. Note that there is more α5-4XR-GFP immunoprecipitated than α5-WT-GFP.

(C) HEp2 cells were transfected with α5-WT-GFP together with HA-Ubiquitin for 24 hr. Fibronectin (FN, 10 μg/ml) and 300 μM leupeptin was added to the cells in serum-free medium for 24 hr, followed by immunoprecipitation with GFP trap beads. Ubiquitination was detected by immunoblotting against anti-HA, and loading was checked using anti-GFP.

(D) Endogenous β1 integrin was immunoprecipitated from BJ fibroblast cell lysates. Immunoblotting was performed using antibodies against Tsg101 and α5 and β1 antibodies. In, input (10%).

(E) α5β1 integrin recycling experiments were performed in BJ cells as described in Experimental Procedures. Error bars represent ± SE of three independent experiments.

(F) Degradation of cell-surface integrin was determined by biotinylating cell-surface proteins and incubating for the different time points indicated. Immunoprecipitation using GFP trap beads was performed at each time point, and all samples were analyzed by western blotting using anti-streptavidin antibody. The graph shows mean values from three independent epxeriments, with error bars representing ±SE. See also Figure S2.

other known interactions and functions of α5 integrin. α5-4XR-GFP was shown to interact with fibronectin and β1 integrin, in the same way as α5-WT-GFP (Figure S2D). We also observed a similar amount of colocalization of α5-WT-GFP or α5-4XR-GFP with Rab21 (Figure S2E), a known interaction partner of α5 integrin (Pellinen et al., 2006), although the extent of colocalization was limited as has been observed before (Hooper et al., 2009). Furthermore, cells expressing α5-4XR-GFP were found to spread on fibronectin to the same degree as α5-WT-GFP-expressing cells (Figure S2F). We also determined whether the expression of α5-4XR-GFP resulted in an alteration of adhesion site turnover. We assessed assembly and disassembly rates of vinculin-RFP in cells transfected with α5-WT-GFP or α5-4XR-GFP and depleted of endogenous α5 integrin. No significant difference in adhesion site turnover was observed (Figure S2G). Therefore, lack of ubiquitination and accompanying endosomal sorting defects seem to be the only consequence of the substitution of the lysine residues in the cytoplasmic tail of α5-WT-GFP.

α5 Ubiquitination Is Required for Sorting of Fibronectin and α5β1 Integrin into the Lumen of MVEs

We used the α5-4XR-GFP construct in order to determine whether ubiquitination of α5 integrin is required for its sorting into MVEs. However, at the light-microscopic level it is difficult to visualize whether a protein localizes to the limiting membrane of the MVE or to its lumen. This problem is circumvented by the use of cells expressing an activated mutant of the small GTPase Rab5 ($Rab5^{Q79L}$), which results in the formation of enlarged endosomal structures due to enhanced homotypic fusion (Stenmark et al., 1994) and has been used to visualize a variety of endosomal proteins (Gillooly et al., 2003; Pelkmans et al., 2004; Raiborg et al., 2002; Simonsen et al., 1998). By electron microscopy, it was shown that many of these enlarged endosomes contain ILVs and are therefore considered as MVEs (Wegner et al., 2009). HEp2-$Rab5^{Q79L}$-expressing cells were transfected with α5-WT-GFP or α5-4XR-GFP, and incubated with or without fibronectin. We observed that α5-WT-GFP was only sorted within the lumen of MVEs upon incubation with fibronectin (Figure 3A), suggesting that fibronectin is required for the sorting of integrins inside MVEs. Results were quantified in several cells and α5-WT-GFP was shown to be significantly sorted within the lumen of MVEs upon incubation with fibronectin (Figure 3C). This was also the case for endogenous α5 and β1 integrin (Figures S3A and S3B and quantified in Figures S3C and S3D). This is consistent with our

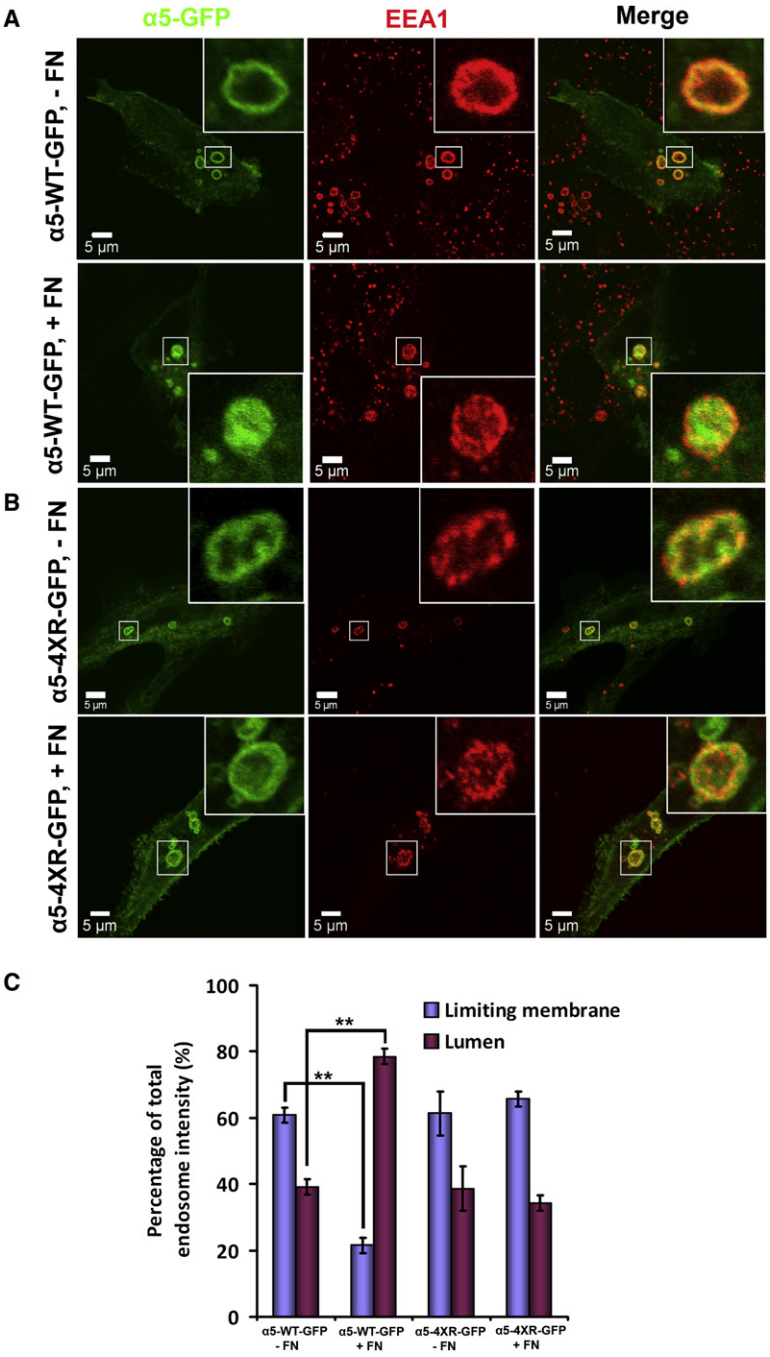

Figure 3. Ubiquitination of α5 Integrin Is Required for Its Sorting into the Lumen of MVEs

HEp2Rab5^{Q79L} cells were transfected with α5-WT-GFP (A) or α5-4XR-GFP (B), serum-starved for 24 hr, and then stimulated with fibronectin (10 μg/ml) together with 300 μM leupeptin for 24 hr. Confocal images are representative of three independent experiments. Scale bar, 5 μm. (C) Localization to the lumen or the limiting membrane of MVEs of α5-GFP constructs with or without fibronectin was quantified in several cells. Error bars represent ±SE of three experiments. **$p < 0.005$. See also Figure S3.

previous results that fibronectin induces ubiquitination of integrins, and therefore their sorting into MVEs. Furthermore, we observed that α5-4XR-GFP was not sorted into the lumen of MVEs even upon incubation with fibronectin (Figure 3B, bottom), consistent with the fact that α5-4XR-GFP cannot be ubiquitinated and therefore cannot be sorted. These results show that ubiquitination of α5 integrin is required for its sorting into MVEs, and that this requires its fibronectin.

In order to determine whether it is in fact the subset of ligand-bound integrins that is sorted to MVEs, we used an antibody that recognizes active β1 integrin, P4G11 (Wayner et al., 1993). We observed colocalization of α5-WT-GFP with P4G11 inside the lumen of enlarged MVEs (Figure S3E), suggesting that integrins localized to MVEs have been activated by binding to fibronectin.

The colocalization of fibronectin with α5β1 integrin within MVEs, and the finding that fibronectin induces α5 ubiquitination, suggests that fibronectin sorting into MVEs requires α5 ubiquitination. To address this directly, endogenous α5 integrin was depleted from HEp2-Rab5^{Q79L} cells, which were retransfected with α5 siRNA-resistant GFP-tagged constructs (WT and 4XR α5 integrin). Localization of endogenous fibronectin was observed by confocal immunofluorescence microscopy. Since HEp2 cells do not produce fibronectin, the fibronectin staining corresponds to matrix-derived fibronectin established by incubating cells with full serum which contains soluble fibronectin, and not due to fibroblast-produced fibronectin. We observed a similar staining pattern between fibronectin and α5-4XR-GFP, which was not sorted within the lumen of MVEs (Figure 4A). Localization was quantified in multiple cells and showed a significant inability of sorting of fibronectin in α5-4XR-GFP-expressing cells (Figure 4B). This suggests that fibronectin requires ubiquitination of α5 integrin at its conserved lysine residues in order to allow its proper sorting, confirming that ligand and receptor are trafficked together.

We next determined whether α5 integrin ubiquitination is required for fibronectin internalization. HEp2 cells were depleted of α5 integrin by siRNA, retransfected with α5-WT-GFP or α5-4XR-GFP, serum-starved and incubated at 4°C with fibronectin, followed by 30 min internalization. Cells were fixed and stained for fibronectin, and internalized fibronectin was quantified. As expected, cells depleted of α5 integrin showed decreased endocytosis of fibronectin (Shi and Sottile, 2008). However, α5 integrin siRNA cells still showed some endocytosed fibronectin, suggesting that there are possibly other α integrins involved in fibronectin endocytosis. These have not been identified so far, and αv integrin inhibitory antibodies had no effect on fibronectin endocytosis (Shi and Sottile, 2008). Cells expressing α5-WT-GFP or α5-4XR-GFP showed similar levels of internalized

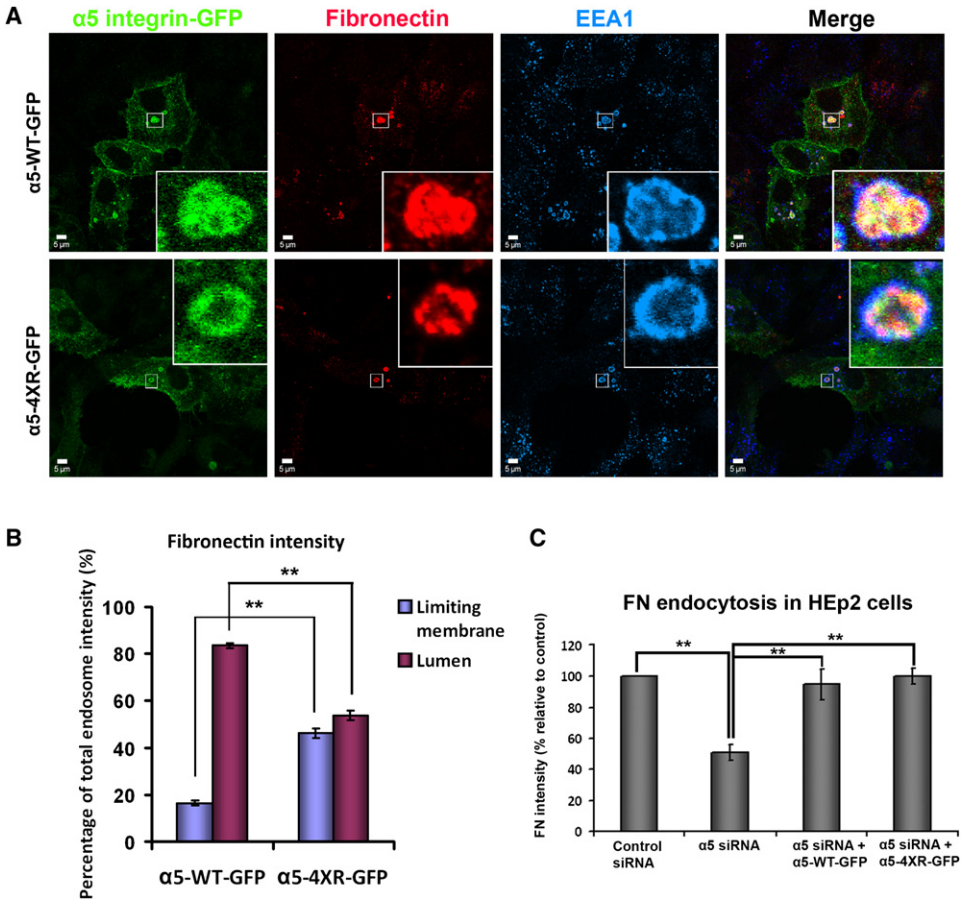

Figure 4. Ubiquitination of α5 Integrin Is Required for Fibronectin Sorting into the Lumen of MVEs, but Does Not Affect Its Endocytosis

(A) HEp2Rab5^{Q79L} were depleted of endogenous α5 integrin by siRNA and retransfected with siRNA-resistant α5-WT-GFP or α5-4XR-GFP. Cells were stained with fibronectin and EEA1. Images are representative of three independent experiments. Scale bar, 5 μm.

(B) Fibronectin localization to the lumen or the limiting membrane of MVEs was quantified in several cells. Error bars represent ±SE of three experiments. **$p < 0.005$.

(C) HEp2 cells were depleted of endogenous α5 integrin by siRNA and retransfected with either siRNA-resistant α5-WT-GFP or α5-4XR-GFP, followed by serum-starvation for 24 hr. Fibronectin (10 μg/ml) was then allowed to bind to the cells for 1 hr on ice, followed by 30 min at 37°C in serum-free medium to allow internalization. Cells were then fixed and stained for fibronectin. Amount of fibronectin internalization was quantified in cells expressing control siRNA, α5 siRNA, or α5-GFP-expressing cells. Error bars represent ±SE of three experiments. **$p < 0.005$.

fibronectin, suggesting that α5 ubiquitination is not required for fibronectin internalization, but only for fibronectin degradation (Figure 4C).

α5β1 Integrin Accumulates in Ubiquitin-Positive Early Endosomes in ESCRT-Depleted Cells

Since ESCRT proteins are involved in the endosomal sorting of ubiquitinated cargoes (Raiborg and Stenmark, 2009), we determined whether the ESCRT machinery is required for the trafficking of integrins. Four ESCRT complexes have been characterized up to date, termed ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III (Raiborg and Stenmark, 2009). It has been established that depletion of key subunits from several ESCRTs yield a more complete phenotype than single depletions (Stuffers et al., 2009b), and we therefore used siRNA against both the ESCRT-0 subunit Hrs and the ESCRT-I subunit Tsg101 to maximize inhibition of the ESCRT machinery. Consistent with previous studies in HeLa cells (Bishop et al., 2002), we observed accumulation of ubiquitinated proteins on endosomal compartments in ESCRT-depleted BJ fibroblasts (Figure 5A). This accumulation did not depend on growth factor stimulation (Figures S4A and S4C), and we hypothesized that it might reflect the accumulation of ubiquitinated integrins. Indeed, we observed a strong accumulation of α5 integrin (Figure 5A, lower panel) and β1 integrin (Figure S4A) at ubiquitin-positive early endosomes in ESCRT-depleted cells. As described previously (Stuffers et al., 2009b), ESCRT depletion caused enlargement of EEA1-positive early endosomes. The colocalizations of α5 and β1 integrin with ubiquitin and EEA1 were quantified, and differences between control and ESCRT siRNA were shown to be statistically significant (Figure 5B; Figure S4B). Because endosomal ubiquitin staining in ESCRT depleted cells occurred even under serum starvation (Figure S4C), ubiquitin accumulation in ESCRT-depleted cells is probably due to other ubiquitinated receptors in addition to integrins. In contrast, the intracellular accumulation of integrins was strongly enhanced in the

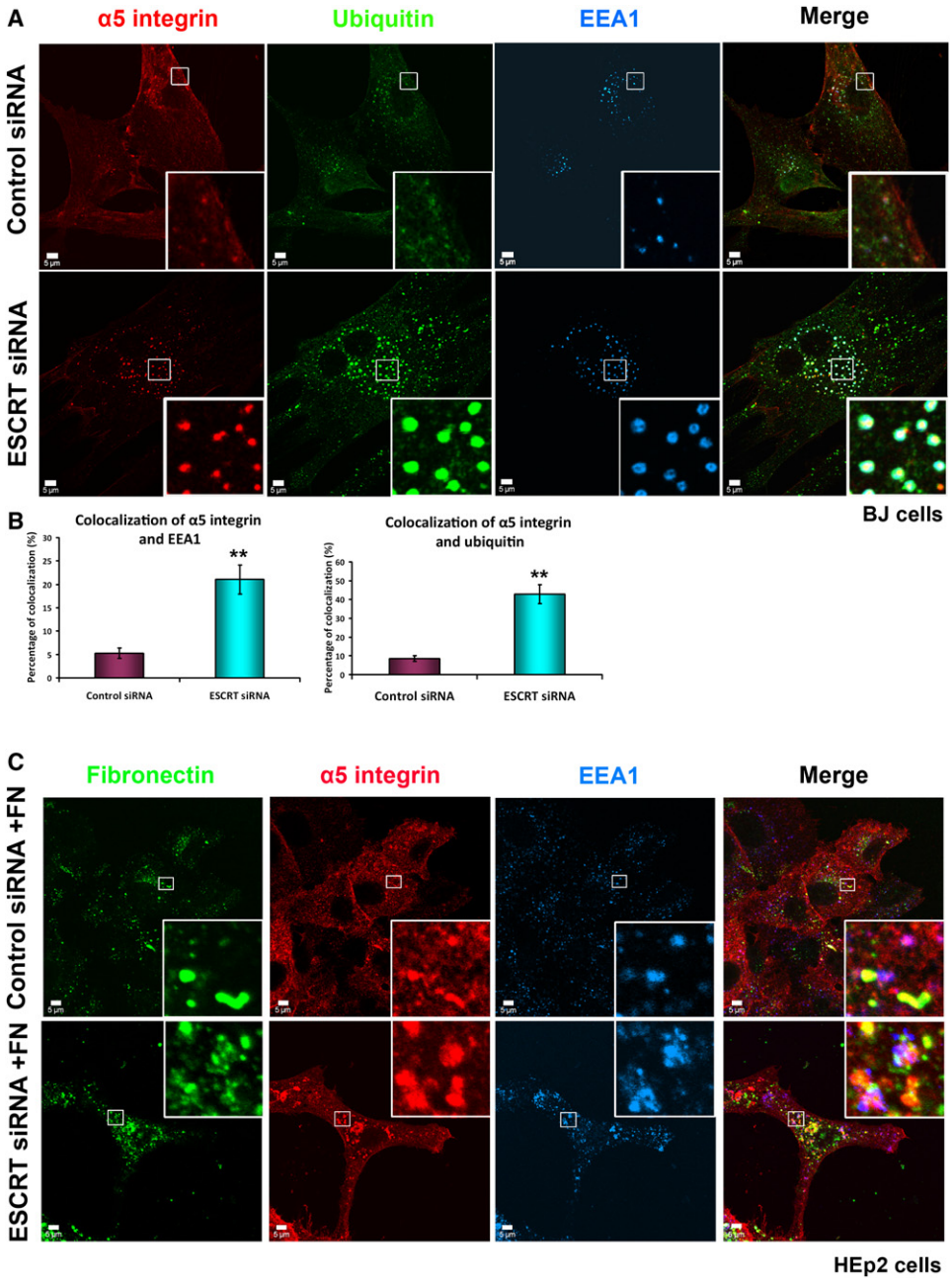

Figure 5. α5β1 Integrin and Fibronectin Accumulate at Ubiquitin-Positive Early Endosomes in ESCRT-Depleted Cells

(A) Confocal images of α5 integrin in control and ESCRT (Hrs and Tsg101)-depleted BJ cells. Images are representative of three independent experiments. Scale bar, 5 μm.

(B) Quantification of colocalization between α5 integrin and ubiquitin or α5 integrin and EEA1. Error bars represent ±SE of three experiments. **$p < 0.005$.

(C) HEp2 cells were depleted of ESCRTs and incubated with fibronectin (10 μg/ml) overnight to allow them to establish a robust fibronectin matrix (pulse), followed by a chase with 300 μM leupeptin for 10 hr. Images are representative of three independent experiments. Scale bar, 5 μm. See also Figure S4.

presence of fibronectin (Figure S4C, quantification in Figure S4D), suggesting that fibronectin is required for the accumulation of integrins with ubiquitin in ESCRT-depleted cells.

In order to determine whether fibronectin trafficking itself was also affected by ESCRT depletion, we depleted HEp2 cells of ESCRTs, serum-starved cells and incubated them overnight with fibronectin, allowing them to establish a robust fibronectin matrix. This was followed by a leupeptin chase in order to conserve the signal. We observed fibronectin labeling between cells and partly colocalizing with α5 integrin in control cells (Figure 5C, upper panel). In ESCRT-depleted cells, we observed higher intensity of fibronectin, suggesting that fibronectin accumulates in these cells and is unable to be degraded. Furthermore, this accumulation occurs at integrin- and EEA1-positive

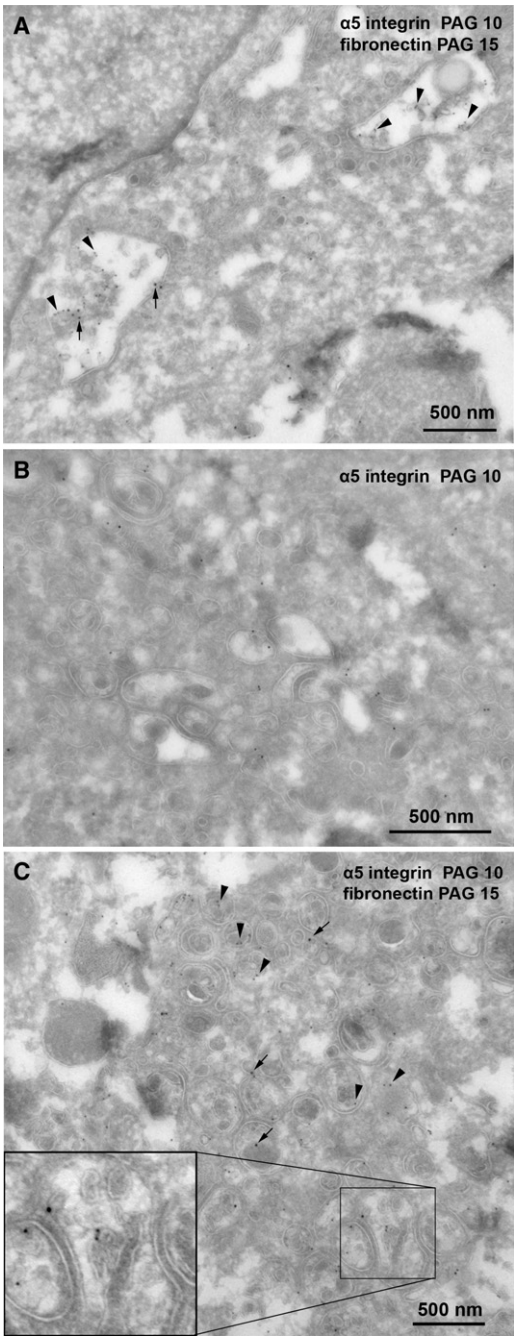

Figure 6. Immuno-Electron Microscopic Localization of Integrin and Fibronectin in ESCRT-Depleted Cells

Single- and double-labeling experiments were performed as described in Experimental Procedures in BJ cells depleted for Hrs and Tsg101 components. α5 integrin was observed in early endosome-like structures (arrowheads in A) and in clusters of smaller endocytic structures (B). Double labeling against α5 integrin (arrowheads) and fibronectin (arrows) demonstrates colocalization of both proteins in the early endosome-like vesicles (A) as well as in the aggregated endocytic vesicles (C). Inset in (C) highlights the ultrastructure of these vesicle clusters, in which the small endosomes seem docked to each other. Scale bar, 500 nm.

structures (Figure 5C, lower panel), suggesting that fibronectin trafficking occurs together with α5 integrin. We therefore conclude that fibronectin trafficking and degradation are also dependent on the ESCRT machinery.

Integrin and Fibronectin Accumulate on Enlarged Endosomes in ESCRT-Depleted Cells

In order to determine the localization of integrin and fibronectin in ESCRT-depleted cells at the ultrastructural level, we performed immuno-EM. Fibronectin and α5 integrin localized to enlarged but otherwise typical early endosomal structures (Figure 6A). We also observed labeling in clusters of smaller endocytic vesicles and tubules that seemed to aggregate in some areas of the cell (Figures 6B and 6C). These structures were strongly reminiscent of previously observed vesiculotubular compartments in ESCRT depleted cells (Doyotte et al., 2005; Razi and Futter, 2006; Stuffers et al., 2009b). This suggests that the enlarged EEA1-positive structures that we observed by immunofluorescence represent both enlarged early endosomes and clusters of vesicles.

Fibronectin and α5β1 Integrin Accumulate at the Protein Level in ESCRT-Depleted Cells

In order to determine whether the overall cellular levels of fibronectin and α5β1 integrin are regulated by lysosomal degradation, we performed western blotting of ESCRT-depleted BJ fibroblasts. Fibronectin and α5 and β1 integrin protein levels accumulated strongly in ESCRT-depleted cells (Figure 7A, quantified in Figure 7B). This could be due to an increase in protein synthesis or a decreased degradation. By quantitative real-time PCR, we investigated whether there were any changes at the mRNA levels of α5 and β1 integrin between control and ESCRT-depleted cells. We could not observe any significant difference in α5 integrin or β1 integrin mRNA levels (Figures 7C and 7D), suggesting that the increase we observed at the protein level was due to a posttranslational event, consistent with inhibited degradation. We also confirmed that ESCRT depletion was nontoxic to the cells by determining that protein synthesis was unaffected (Figure S5).

Ubiquitination of α5 Integrin Is Required for Proper Fibroblast Migration

In order to determine the functional significance of the observed ubiquitination of α5β1 integrin, we performed cell migration assays in cells depleted of endogenous α5 integrin (Figure 7E), or retransfected with α5-WT-GFP or α5-4XR-GFP. We performed live-cell time-lapse microscopy over a period of 5 hr to observe differences in cell velocity. As expected (Bauer et al., 1993), we observed a decrease in cell velocity in cells that had been depleted of α5 integrin, and normal velocity was restored upon transfection with siRNA-resistant α5-WT-GFP (Figure 7F). Importantly, such rescue was not observed when α5-depleted cells were transfected with the 4XR mutant version of the protein, which cannot be ubiquitinated and sorted into MVEs. This result directly demonstrates that ubiquitination of α5 integrin, which mediates its ESCRT-dependent endosomal sorting, is required for cell migration.

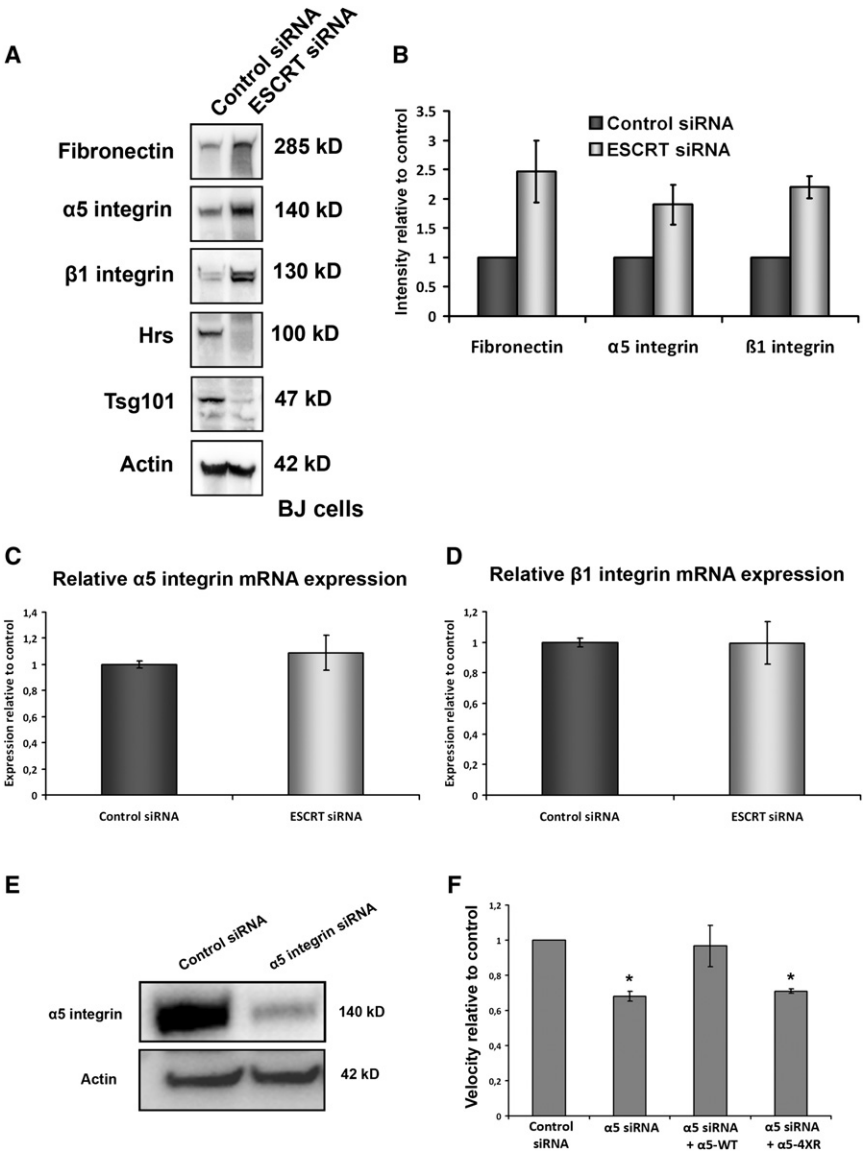

Figure 7. Fibronectin and α5β1 Integrin Accumulate at the Protein Level in ESCRT-Depleted Cells, and α5 Integrin Ubiquitination Is Required for Cell Migration

(A) Western blotting of control and ESCRT-depleted BJ cells.

(B) Quantification of fibronectin and α5β1 integrin levels in control and ESCRT-depleted cells. Quantifications are based on three independent siRNA and western blotting experiments and were performed using Quantity One Software (Biorad). Error bars represent ±SE of three experiments.

(C and D) RNA isolation, cDNA synthesis, and quantitative real-time PCR of α5 and β1 integrin were performed in control siRNA- and ESCRT siRNA-transfected cells. Data are representative of three independent experiments where efficient knockdown of the ESCRT components was verified by western blotting. Error bars represent ±SE of three experiments.

(E) Western blotting of α5 integrin depletion in BJ fibroblast cells.

(F) Quantification of velocity in control siRNA-transfected cells and α5 integrin siRNA-transfected cells, which have been retransfected with siRNA-resistant α5-WT-GFP or α5-4XR-GFP. Error bars represent ± SE of three experiments. *$p < 0.05$. See also Figure S5.

DISCUSSION

While endocytic recycling of integrins plays a well-documented role in cell migration (Caswell and Norman, 2006; Pellinen and Ivaska, 2006; Ridley et al., 2003), the degradative endocytic sorting of integrins has received little attention so far. In this study, we present evidence that a fraction of α5β1 integrin is degraded together with fibronectin in a ligand- and ESCRT-dependent manner, and that this is required for proper fibroblast migration. Indirect evidence has previously suggested that α5 integrin is ubiquitinated in response to fibroblast growth factor receptor 2 activation, resulting in its proteosomal degradation (Kaabeche et al., 2005). Moreover, the platelet integrin $\alpha_{IIb}\beta_3$ has been shown to interact with the E3 ubiquitin ligase RN181 (Brophy et al., 2008). Additionally, integrin-associated proteins have been shown to be regulated by ubiquitination. The signal transducing adaptor STAP-2 associates with FAK, promoting its proteasomal degradation by recruiting the E3 ubiquitin ligase Cbl (Sekine et al., 2007). Furthermore, it was recently shown that talin, an integrin and actin-binding protein, is ubiquitinated by the E3 ubiquitin ligase Smurf1, which results in proteosomal degradation of the talin head (Huang et al., 2009). It is interesting to note that ubiquitination of E-cadherin, one of the best-studied components of adherens junctions, is essential for its sorting to the lysosome, and that this is mediated by the ESCRT machinery (Palacios et al., 2005). Thus, the present work fits into the picture of ubiquitin as a crucial regulator of cell adhesion and associated processes.

Because it has been documented that α5β1 integrin recycles via the PNRC (Roberts et al., 2001), we measured recycling from this compartment. Consistent with previous reports in other cell types, integrin recycles fairly rapidly in BJ fibroblasts, with a halftime of less than 1 hr. In contrast, the degradation we observed by following a total pool of cell-surface integrins has a half-life of several hours. The reason why total integrin degradation is slower than recycling is probably due to the fact that only a minor pool of cell-surface integrin molecules is targeted to the degradative pathway. Therefore, it is possible that fibronectin-bound integrin is in fact trafficked faster than the indicated half-life to lysosomes, but in order to detect this we would need to be able to detect ligand-bound fibronectin. We hypothesize that the degraded pool of integrins corresponds to the active integrin molecules, which have been activated by binding

to fibronectin. In support of this hypothesis, we observed activated integrin molecules within the lumen of MVEs as well as frequent colocalization of fibronectin and integrin in MVEs by EM. We also observed ubiquitination of α5 integrin and sorting of integrins into the lumen of MVEs exclusively in response to fibronectin binding. Consequently, the intracellular accumulation of integrins in ESCRT-depleted cells was also enhanced in cells stimulated with fibronectin. The integrins that we observed in early endosomes void of fibronectin by EM might represent nonactivated integrin molecules that are destined for recycling back to the plasma membrane, and these might correspond to the constitutively endocytosed integrins (Bretscher, 1989, 1992; Sczekan and Juliano, 1990). In contrast, we propose that degradation is a stimulated process, dependent on fibronectin.

Mutagenesis of the cytosolic lysines of α5 integrin was confirmed to specifically affect its ability of being ubiquitinated. Since these substitutions are conservative (lysine to arginine in all cases), they are unlikely to disturb salt bridges between α and β subunits. It should be noted, however, that α cytoplasmic tails have conserved GFFKR sequences, which when deleted result in locking integrins in a high affinity state (Crowe et al., 1994; Hughes et al., 1995; O'Toole et al., 1991, 1994). Furthermore, a nonconservative substitution of phenylalanine to alanine (GFFKR to GAFKR) also results in activation of the integrin (Hughes et al., 1996). Thus, although a conservative GFFKR to GFFRR substitution is unlikely to affect the activation of α5 integrin, this possibility cannot be ruled out completely.

The 4K→R substitutions had a strong effect on the localization and trafficking of α5 integrin and fibronectin. Neither protein was able to be sorted within the lumen of MVEs when the ubiquitination-defective α5 mutant was expressed. Matrix fibronectin accumulation was observed at early endosomal structures in ESCRT-depleted cells, similarly to α5β1 integrin, confirming that ligand and receptor are indeed trafficked via the same intracellular route, which is dependent on the ESCRT machinery. EM revealed that the structures observed by immunofluorescence are probably clusters of vesicles as well as enlarged endosomes, which are typically observed in ESCRT-depleted cells (Doyotte et al., 2005; Razi and Futter, 2006; Stuffers et al., 2009b). Our findings provide a mechanistic explanation for the previous observation that matrix fibronectin is degraded in lysosomes (Ray et al., 2006).

Inhibition of the expression of subunits of the ESCRT machinery had a marked effect on the expression levels of integrin proteins. This was observed not to be due to a difference in mRNA expression, but rather to decreased protein degradation. ESCRT proteins have been reported to have an important role in signal attenuation of several cell-surface receptors, by recognizing their ubiquitinated cytoplasmic tails and sorting them into ILVs of MVEs (Raiborg and Stenmark, 2009). After fusion of the MVEs with lysosomes, both ILVs and their protein content are degraded by lipases and proteases (Gruenberg and Stenmark, 2004; Katzmann et al., 2002). An increase in integrin levels after ESCRT depletion therefore suggests that integrins, similarly to growth factor receptors, are down-regulated by the MVE pathway. We show that integrin trafficking is arrested at early endosomal structures when ESCRT proteins are depleted, and that this results in impaired trafficking toward late endosomes/lysosomes. Our results indicate that a fraction of α5β1 integrin is recognized by the ESCRT machinery presumably through engagement of ubiquitin moieties appended to α5, and that this results in lysosomal degradation of the fibronectin receptor. At this point, we cannot exclude the additional possibility that the ILVs of MVEs positive for α5β1 integrin might be shed as exosomes, which could transfer integrin molecules to the same or neighboring cells via the extracellular medium. It has indeed been described that some exosomes positive for integrins are released in the extracellular medium (Rieu et al., 2000).

Our findings have revealed an unanticipated role for integrin degradation in cell migration. We also observed that mitogenic stimuli caused increased mRNA levels of α5 integrin, β1 integrin, and Hrs in fibroblasts (data not shown), consistent with the possibility that increased integrin turnover during cell migration needs to be compensated by higher synthesis rates. But why is integrin turnover so important for cell migration? The mechanism suggested by this study is that matrix-derived fibronectin requires α5 integrin ubiquitination in order to be sorted within the lumen of MVEs on its way to lysosomal degradation. Since both integrin degradation (this study) and fibronectin degradation (Hocking and Chang, 2003) are required for cell migration, a possible mechanism by which α5-4XR-GFP is unable to rescue the velocity of α5 integrin-depleted cells is that fibronectin-bound integrin molecules accumulate in endosomes, and if recycled, would result in the formation of dysfunctional adhesion sites, resulting in increased adhesion and buildup of extracellular matrix, thereby hindering cell migration. Another possible scenario is that degradation is required for proper attenuation of integrin signaling that impacts on migration (Huveneers and Danen, 2009). An interesting parallel to our observations is the recent finding that ubiquitin-dependent proteasomal degradation of another adhesion site component, talin, is required for cell migration (Huang et al., 2009). It is striking that two different constituents of adhesion sites both are turned over by ubiquitin-dependent processes that are required for cell migration, although their mechanisms are completely different.

Endocytosis of α5β1 integrin has been reported to be constitutive (Bretscher, 1989; Sczekan and Juliano, 1990), and our studies confirm that α5β1 integrin is efficiently endocytosed even in the absence of fibronectin. We thus conclude that fibronectin-stimulated ubiquitination of α5 controls the endosomal sorting, but not the endocytosis of α5β1 integrin. One might suppose that the E3 ligase(s) responsible for the ubiquitination might interact with the activated integrins only, which have a different conformation than the non-matrix-bound integrins. It must also be determined whether other integrin proteins are ubiquitinated and sorted into MVEs for degradation to lysosomes in the same way as α5β1 integrin, or whether this is specific to the fibronectin receptor. For example, vitronectin is also trafficked to lysosomes (Memmo and McKeown-Longo, 1998), raising the possibility that the vitronectin receptor, αvβ3 integrin, might also be degraded via the lysosomal pathway. Finally, it needs to be established whether the effect on cell migration is specific to fibroblasts or whether this knowledge can also be extended to other cell types. In order to push the parallel between EGFR trafficking and α5β1 integrin even further, one might investigate whether fibronectin itself stimulates MVE formation, in a similar way to EGF (White et al., 2006). Knowing that both integrin and ESCRT expression can be affected in

cancer cells (Janes and Watt, 2006; Stuffers et al., 2009a), it will be especially interesting to learn whether metastatic cancer cells have acquired mechanisms that overcome the requirement for ESCRT-mediated integrin degradation in cell migration.

EXPERIMENTAL PROCEDURES

RNA Interference

BJ cells were plated the day before transfection and transfected with 75 nM siRNA against Hrs (sense CGACAAGAACCCACACGUC and antisense GACGUGUGGGUUCUUGUCG), 10 nM Tsg101 siRNA (sense CCGUUUAGAU CAAGAAGUAUU and antisense AAUACUUCUUGAUCUAAACGG), or 85 nM nontargeting siRNA (Dharmacon). Transfection was performed using RNAi Max lipofectamine (Invitrogen) according to the manufacturer's specifications. Cells were replated after 3 days and grown for another 2 days before they were used for experiments. For α5 siRNA depletion, 50 nM siRNA (QIAGEN) against α5 integrin was transfected for a period of 2 days.

Confocal Microscopy

Cells were permeabilized with 0.05% saponin in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$ [pH 6.8]) for 5 min prior to a 15 min fixation in 3% paraformaldehyde. Primary antibodies were diluted in PBS supplemented with 0.05% saponin. Confocal images were acquired with a 100× objective on a Zeiss LSM 510 Meta confocal laser-scanning microscope. Analysis of colocalization was done using LSM Image Examiner software.

Surface Half-Life of Integrins

It has previously been established that it is possible to determine surface half-life of integrins by biotinylation (Witkowski et al., 2000). In brief, BJ fibroblasts or transfected HEp2 cells were surface-biotinylated with sulfo-NHS-LC-biotin and incubated for 0, 12, 18, or 24 hr with full medium, followed by lysis and immunoprecipitation with α5 integrin antibody (BJ samples) or GFP trap (HEp2 samples). Samples were analyzed by SDS-PAGE, transferred to PVDF membrane and blotted with streptavidin-HRP antibody.

For additional Experimental Procedures, please refer to the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2010.06.010.

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